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Somatostatin receptor-2 negatively regulates β-adrenergic receptor mediated Ca^{2+} dependent signaling pathways in H9c2 cells

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ABSTRACT

In the present study, we report that somatostatin receptor 2 (SSTR2) plays a crucial role in modulation of β_1AR and β_2AR mediated signaling pathways that are associated with increased intracellular Ca^{2+} and cardiac complications. In H9c2 cells, SSTR2 colocalizes with β_1AR or β_2AR in receptor specific manner. SSTR2 selective agonist inhibits isoproterenol and formoterol stimulated cAMP formation and PKA phosphorylation in concentration dependent manner. In the presence of SSTR2 agonist, the expression of PKCα and PKCβ was comparable to the basal condition, however SSTR2 agonist inhibits isoproterenol or formoterol induced PKCα and PKCβ expression, respectively. Furthermore, the activation of SSTR2 not only inhibits calcineurin expression and its activity, but also blocks NFAT dephosphorylation and its nuclear translocation. SSTR2 selective agonist abrogates isoproterenol mediated increase in cell size and protein content (an index of hypertrophy). Taken together, the results described here provide direct evidence in support of cardiac protective role of SSTR2 via modulation of Ca^{2+} associated signaling pathways attributed to cardiac hypertrophy.

1. Introduction

Adrenergic receptors (ARs) are the prominent and most studied members of G-protein coupled receptor (GPCR) family and have been well characterized for their crucial role in cardiovascular complications [1,2]. The activation of β_1 and β_2AR is associated with different pathophysiological responses and illicit receptor specific role in heart failure [3–6]. Several studies have described the activation of second messenger cyclic-adenosine monophosphate (cAMP) by β_1 and β_2AR in receptor specific manner [7]. Furthermore, cAMP dependent protein kinase (PKA) induced the phosphorylation of phospholamban and cardiac contractile protein via activation of β_1AR but not of β_2AR [8]. In addition, over-expression of β_1AR also induced hypertrophy and apoptosis in cardiomyocytes [9,10]. Receptor coupling to different G proteins i.e., Gs and Gi is important in signal transduction [11–14]. β-ARs coupling to Gs protein, regulation of adenylyl cyclase (AC) mediated cAMP/PKA and consequent changes in Ca^{2+} related signaling cascades exert key roles in cardiac contraction. Most importantly, Ca^{2+} mediated regulation of myocyte contraction is intimately associated with β-ARs coupling to AC. In addition to the changes in PKC isoforms, the best studied process that serves in promoting cardiac hypertrophy is linked with the Ca^{2+} regulated calcineurin dependent nuclear factor of activated T-cells (NFAT), dephosphorylation and its nuclear translocation.

The role of somatostatin (SST) in pathophysiological conditions including neurological diseases, pituitary tumor, and breast cancer as well as tumors of different origins is well established and undisputed [15–19]. However, studies supporting the role of SST in the cardiovascular system such as in hypertrophy and contractile functions are limited [20–22]. Furthermore, the clinical implication and physiological significance of SST in the cardiovascular system have not been well appreciated yet. We recently described the role of SST in the regulation of β-AR mediated signal transduction in H9c2 cells [19]. SST binds to and acts on five different receptor subtypes (SSTR1–5) which belong to GPCR family and have been well characterized pharmacologically in various target tissues [15]. These observations raise the question whether SST modulates β-AR stimulated changes in signaling pathways directly via activation of SSTR subtypes or indirectly via regulation of hormones or growth factors to alleviate the loss of cardiac function is not well understood. While SST plays a critical role in cardiac hypertrophy and heart failure, receptor subtype associated with such role remains elusive [22–26].

SSTR subtypes are present in variable density in cardiac cells or heart tissues whereas very little is known pertaining to their function in cardiac complications. SSTR2 induces apoptosis (cytotoxic effect) and cell growth arrest (cytostatic effect) in cell specific manner and involves in the regulation of Ca^{2+} homeostasis. Although, there is no direct evidence of SSTR2 mediated regulation of L-type Ca^{2+} channels in cardiac cells, SSTR2-selective agonists like MK 678 and BIM 23027 inhibit Ca^{2+} conductance in mouse anterior pituitary cells (AtT-20) that predominantly expresses L-type Ca^{2+} channels [27]. Similarly, rat SSTR2 upon

Abbreviations: AC, adenylyl cyclase; β-AR, β-adrenergic receptor; Ca^{2+}, calcium; cAMP, cyclic adenosine monophosphate; FSK, forskolin; LVH, left ventricular hypertrophy; NFAT, nuclear factor of activated T-cells; PKA, Protein Kinase A; PKC, Protein Kinase C; SSTR2, somatostatin receptor 2

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activation couples with Ca^{2+} channels and leads to inhibition of Ca^{2+} influx in RIN cells [28]. Given the ability of SSTR2 to inhibit Ca^{2+} influx, we speculate that SSTR2 activation could be beneficial in cardiomyopathy specifically via modulation of Ca^{2+} associated pathways which are triggered by β_1 and β_2AR. To test this hypothesis, instead of directly measuring the changes in intracellular Ca^{2+} levels, the present study was undertaken to determine the role of SSTR2 in modulation of β_1 and β_2AR mediated Ca^{2+} associated signaling pathways including cAMP/PKA, PKCα/β, calcineurin and NFAT which functions in articulated manner in cardiac tissue. We also analyzed the changes in cell size and total protein content in response to the activation of β-ARs and SSTR2. The results described here uncovered the crucial role of SSTR2 in the regulation of signaling pathways with potential therapeutic intervention in regulating factors contributing in cardiac complications including cardiomyopathy and heart failure.

2. Materials and methods

2.1. Chemicals and reagents

H9c2 rat fetal cardiomyocytes were kindly provided by Dr. David Fedida, UBC. SSTR2 agonist L-779976 was kindly obtained from Dr. SP Rohrer, MERCK. Isoproterenol hydrochloride, formoterol hemifumarate, CGP 20712 dihydrochloride and ICI-118,551 hydrochloride were purchased from TOCRIS Bioscience (Ellisville, MO). Antibody against β-actin was purchased from Sigma-Aldrich, Inc., St. Louis, MO. Anti-β_1AR goat polyclonal antibody was purchased from TOCRIS Bioscience (Ellisville, MO) and mouse monoclonal β_2AR antibody was purchased from Abcam (Cambridge, MA). Rabbit polyclonal antibodies directed against phospho and total-PKA, NFAT and PKCβ were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibodies against phospho- and total-ERK1/2 and anti-PKCα were purchased from Cell Signaling Technology (Danvers, MA). Mouse monoclonal antibody against calcineurin was procured from BD Laboratories. Fluorescein/Cy3 and HRP-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). cAMP assay kit was purchased from BioVision, Inc. CA, USA. Protein A/G-Agarose beads were purchased from Calbiochem, EMD Biosciences (Darmstadt, Germany). Calcineurin assay kit was procured from EMD Millipore (Darmstadt, Germany). Reagents for electrophoresis were purchased from Bio-Rad Laboratories (Mississauga, ON). Dulbecco's modified Eagle's medium, Trypsin–EDTA, and Dulbecco's Phosphate Buffered Saline (D-PBS) were purchased from Gibco, Invitrogen (Burlington, ON, Canada). Other reagents of AR grade were procured from various sources.

2.2. Cell culture

H9c2 rat fetal cardiomyocytes were grown in Dulbecco's modified Eagle's high glucose medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin under humid atmosphere at 37 °C. Cell cultures between passages 3 to 5 at 70–80% confluency were used for each experiment.

2.3. Immunocytochemistry

SSTR2 and βAR subtype colocalization in H9c2 cells was determined as described earlier [29,30]. Briefly, cells were grown on poly-D-Lysine coated coverslips and fixed with 4% paraformaldehyde for 20 min on ice. After three washes with PBS, the cells were incubated with 5% normal goat serum in PBS for 1 h at room temperature followed by overnight incubation with antibodies for SSTR2 (1:500) and β_1AR or β_2AR (1:300) in 1% normal goat serum in PBS at 4 °C. Following three subsequent washes, the cells were then incubated with Cy3 and/or FITC conjugated secondary antibodies for 1 h at room temperature. Coverslips were mounted onto microscope slides and images were observed under Leica Confocal microscope. For NFAT expression, H9c2 cells were treated with receptor specific agonist alone or in combination for 30 min at 37 °C and processed for immunocytochemistry using NFAT specific antibody.

2.4. Co-immunoprecipitation and Western blot analysis

H9c2 cells were treated with L-779976, isoproterenol or formoterol alone or in combination for 30 min at 37 °C and cell membrane was prepared in homogenizing buffer as previously described [31]. Membrane pellets were washed and resuspended in 20 mM Tris–HCl, pH 7.5 in the presence of protease inhibitors (1:100). Samples were incubated with β-AR specific antibody (1:500) overnight at 4 °C on the rocking shaker. 25 μl of protein A/G-agarose beads was added to each tube to immunoprecipitate antibody for 2 h at 4 °C. Beads were then washed three times in PBS and solubilized in Laemmli sample buffer (Bio-Rad) containing 5% β-mercaptoethanol. The fractionated proteins were transferred to a 0.2 μM nitrocellulose membrane in transfer buffer. Membranes were blotted with anti-SSTR2 antibody (dilution 1:500) for the detection of SSTR2. For Western blot analysis, H9c2 cells were treated with L-779976 (10 nM) for 5, 10, 20 and 30 min or with different concentrations of L-779976 (1, 10 and 20 nM) for 15 min at 37 °C. In addition, H9c2 cells were also treated with isoproterenol or formoterol (1 μM) in combination with increasing concentrations of L-779976 (0–50 nM) for 15 min at 37 °C. Treatments were terminated by adding ice-cold PBS and cells were lysed by using RIPA buffer (containing 50 mM Tris–HCl, 150 mM sodium chloride, 1% igepal CA-630, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS), pH 8.0). Equal amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TBS-T (pH 7.6, 20 mM Tris HCl, 137 mM NaCl and 0.2% Tween 20) and subsequently incubated with polyclonal antibody against phospho or total-ERK1/2, PKA or NFAT and PKCα and β at 4 °C overnight. After washing, the membrane was then incubated with HRP-conjugated secondary antibodies and detected by using enhanced chemiluminescence detection reagent (GE Healthcare, Baie d'Urfe QC) according to the manufacturer's instructions.

2.5. Adenylyl cyclase activity assay

H9c2 cells were grown till >70% cell confluency in 6 well culture plates and cAMP levels were estimated by enzyme linked immunosorbent assay (ELISA). Briefly, cells were incubated for 30 min in the presence of 20 μM forskolin (FSK) and 0.5 mM 3-isobutyl-1-methylxanthine with receptor specific agonist L-779976, isoproterenol, formoterol, CGP 20712 and ICI 118,551 alone or in combination at 37 °C. cAMP was determined by immunoassay using a cAMP Kit from BioVision, Inc. CA, USA according to the manufacturer's guidelines.

2.6. Calcineurin activity assay

Colorimetric assay for measuring cellular calcineurin (PP-2B) phosphatase activity was performed as per the manufacturer's instruction. Briefly, H9c2 cells were treated with L-779976, isoproterenol and formoterol alone or in combination for 30 min at 37 °C. Cells were lysed by using lysis buffer (provided in the kit) and high speed supernatant was further desalted using pre-packed columns (Bio-Rad Laboratories). Total protein content in the samples was determined by using Bradford assay and samples were normalized. ELISA was performed to determine the phosphate released by calcineurin phosphatase as per the manufacturer's instructions. Calcineurin activity was calculated as [Calcineurin Activity = Total Activity − Activity in the presence of EGTA Buffer] and/or [Calcineurin Activity = Activity in the presence of Okadaic Acid − (Activity in the presence of Okadaic Acid + EGTA)].

2.7. Cell size and total protein content analysis

H9c2 cells grown to 60–70% confluency were serum deprived for 24 h and treated with SSTR2 agonist L-779976 (500 nM) and β_1AR agonist isoproterenol (10 μM) alone or in combination for 48 h in the presence of 1% FBS [32]. Cells were imaged by using the IncuCyte Zoom system from Essen Bioscience. Images were captured every 2 h interval for 48 h using a 10× objective. For total protein content, cells were treated as indicated in the presence of 5% FBS and trypsinized cells were centrifuged and lysed with 100 μl lysis buffer. Protein concentration in total cell lysates was measured using the Bradford assay (Sigma-Aldrich, Inc., St. Louis, MO) with bovine serum albumin as standard. Protein content per cell was determined by dividing the total protein with the cell number for each condition.

2.8. Statistical analysis

The data was analyzed using GraphPad Prism 4.0. The data was statistically analyzed using one-way ANOVA and the post-hoc Dunnett's or Bonferroni's test was applied according to the experimental conditions to compare treatments. Significant statistical differences were taken at p values <0.05. Results are presented as mean ± SD unless otherwise stated.

3. Results

3.1. Colocalization of somatostatin receptor 2 and β-adrenergic receptors in H9c2 cells

As shown in Fig. 1A and B, H9c2 cells exhibit relatively higher expression of β_2AR than β_1AR. Receptor like immunoreactivity was predominantly confined intracellularly with strong expression in perinuclear zone. Like βARs, SSTR2 like immunoreactivity was also observed intracellularly. β_1AR (A) and β_2AR (B) displayed variable degree of colocalization with SSTR2 in H9c2 cells. β_1AR colocalized with SSTR2 strongly in perinuclear zone with weak colocalization in the rest of the intracellular regions. SSTR2 and β_2AR exhibited widespread cytoplasmic colocalization. Most importantly, no uniform pattern of colocalization between SSTR2 and β-AR subtypes was observed. As illustrated, some cells displayed strong colocalization while other cells were either devoid or exhibit weak colocalization.

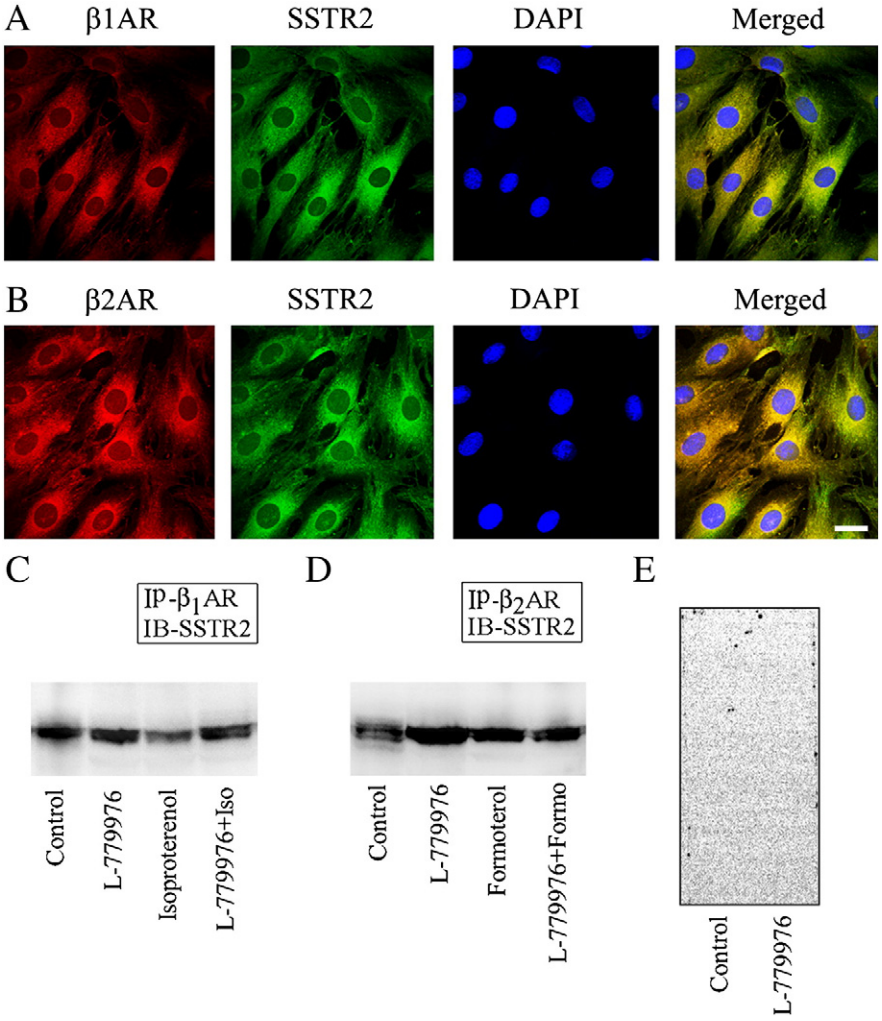

Fig. 1. Interaction between SSTR2 and β_1AR or β_2AR in H9c2 cells. Immunofluorescence photomicrographs illustrating the colocalization of SSTR2 with β_1AR or β_2AR in H9c2 (A and B). β_1AR or β_2AR positive H9c2 cells are shown in red color in panels A and B. SSTR2 like immunoreactivity in H9c2 cells is shown in green and cells displaying colocalization are indicated by yellow or orange color in merged images (panels A and B). DAPI represents nuclear staining in blue color in both panels. Note the significantly variable intensity of receptor expression with strong colocalization between SSTR2 and β-ARs in perinuclear region of the cells. SSTR2 was highly expressed in β_1AR (C) and β_2AR (D) immunoprecipitates prepared from control and treated cells as indicated. Note the significant loss of SSTR2 expression in β_1AR immunoprecipitate upon treatment with isoproterenol in comparison to control (C). In contrast, SSTR2 expression was increased in β_2AR immunoprecipitate upon SSTR2 specific agonist L-779976 and/or formoterol treatment (D). No expression of SSTR2 at the expected molecular weight was observed in empty beads (in the absence of β-AR antibodies) indicating the specificity of interaction (E). Scale bar = 10 μm.

3.2. Expression of SSTR2 in β-AR immunoprecipitate in H9c2 cells

We next determined the expression of SSTR2 in β_1AR (Fig. 1C) and β_2AR (Fig. 1D) immunoprecipitates to anticipate any possible interaction between these receptors. H9c2 cells were treated with SSTR2 and β_1AR or β_2AR selective agonist alone or in combination for 30 min at 37 °C. Membrane fraction prepared was immunoprecipitated with specific antibodies against β_1AR or β_2AR and immunoblotted with antibody against SSTR2. As shown in Fig. 1C, SSTR2 was highly expressed in β_1AR immunoprecipitate prepared from control and treated cells at the expected molecular size of >100 kDa. SSTR2 expression in β_1AR immunoprecipitate was comparable to basal expression upon treatment with L-779976 alone or in combination with isoproterenol however relatively lower in cells treated with isoproterenol.

In comparison to β_1AR immunoprecipitate, the expression of SSTR2 (>100 kDa) was comparatively weak in β_2AR immunoprecipitate prepared from control cells, whereas increased upon treatment with L-779976 (Fig. 1D). In contrast, treatment with formoterol alone or in the presence of L-779976 displayed comparable SSTR2 expression albeit relatively higher than control. To ascertain the specificity of Co-IP membrane fraction incubated in the absence of primary antibodies or blank beads were devoid of SSTR2 expression (Fig. 1E). These results are possible indication in support that SSTR2/β-ARs might exist in a heteromeric complex in H9c2 cells.

3.3. SSTR2 mediated inhibition of cAMP is enhanced in the presence of β-AR antagonist

We recently described that SST inhibits β_1/β_2AR stimulated cAMP in H9c2 and HEK-293 cells cotransfected with SSTR5 and βARs [19,29,30]. Interestingly, SST mediated inhibition of cAMP was further enhanced in the presence of β_1AR or β_2AR antagonist [19,29,30]. Accordingly, we next studied whether inhibitory effect of SST on FSK stimulated cAMP is mediated by SSTR2 in H9c2 cells. Cells were treated with SSTR2 specific agonist L-779976, isoproterenol and formoterol alone or in combination for 30 min at 37 °C in the presence of FSK and processed for cAMP levels. As illustrated in Fig. 2, FSK stimulated cAMP was inhibited by 19.51 ± 0.57% in the presence of SSTR2 agonist (10 nM). Conversely, FSK stimulated cAMP was significantly enhanced by 29.42 ± 0.88% and 66.16 ± 2.3% in the presence of β_1AR and β_2AR specific agonists respectively. SSTR2 agonist in combination with isoproterenol or formoterol resulted in significant attenuation in β-AR induced cAMP levels in H9c2 cells (4.04 ± 0.85% and 39.65 ± 0.82%) respectively. Of note, FSK stimulated cAMP was unaffected in the presence of β-AR specific antagonist, however, L-779976 mediated inhibition of FSK stimulated cAMP was significantly enhanced in the presence of β-AR antagonist. As illustrated in Fig. 2, in the presence of SSTR2 agonist in combination with ICI or CGP, FSK stimulated cAMP was inhibited by 20.76 ± 2.5% and 30.90 ± 2.5% respectively. This inhibition was significantly different in the presence of SSTR2 agonist with ICI in comparison to CGP selective antagonist for β_1AR and β_2AR respectively. These results suggest that blocking of β-ARs potentiates SSTR2 coupling to AC with pronounced inhibitory effect on cAMP formation.

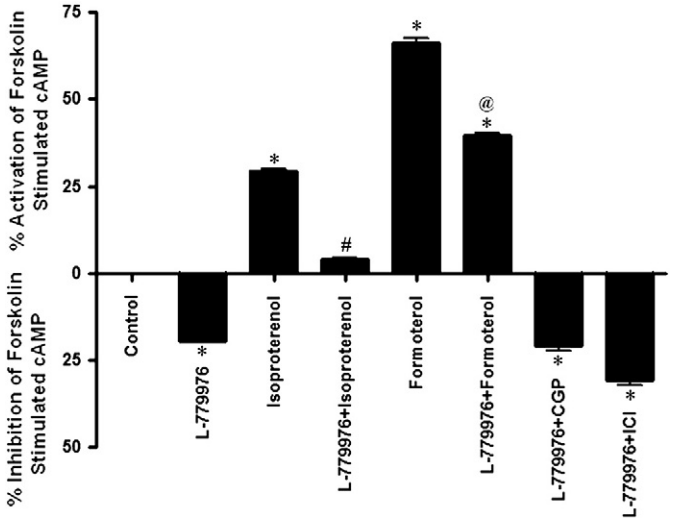

Fig. 2. Receptors coupling to adenylyl cyclase. H9c2 cells were incubated with 20 μM FSK with or without SSTR2, β_1AR and β_2AR agonists or antagonists alone or in combination at 37 °C for 30 min. cAMP formation was inhibited in the presence of SSTR2 agonist and enhanced upon treatment with β-AR agonists. Increased cAMP formation induced by β-AR agonists was significantly abrogated in the presence of SSTR2 agonist. Note the significantly augmented effect of SSTR2 on the inhibition of FSK stimulated cAMP upon β_2AR inactivation by antagonist ICI. Data are shown as % inhibition/activation of FSK stimulated cAMP formation. Data analysis was done by using ANOVA and post-hoc Bonferroni's to compare against basal (*, $p < 0.05$), isoproterenol treatment (#, $p < 0.05$) and formoterol treatment (@, $p < 0.05$); n = 3.

3.4. Concentration and time dependent effects of SSTR2 activation on PKA phosphorylation

As reported previously, changes in cAMP and intracellular Ca^{2+} levels are directly associated with the activation of PKA. Therefore, we next determined the status of PKA phosphorylation in H9c2 cells. SSTR2 specific agonist L-779976 displayed time and concentration dependent inhibitory effects on PKA phosphorylation (Fig. 3A). Despite higher PKA activation in basal condition, L-779976 (10 nM) inhibited PKA phosphorylation significantly at 5, 10 and 20 min treatments, which was recovered to basal level following prolonged treatment for 30 min. As shown in Fig. 3A, cells treated with 1, 10 and 20 nM of L-779976 for 15 min at 37 °C resulted in significantly lower phospho-PKA levels in comparison to control without any noticeable changes at different concentrations.

To determine whether activation of SSTR2 negatively regulates β_1AR or β_2AR mediated PKA phosphorylation, cells were treated with SSTR2 agonist L-779976 (1–50 nM) in combination with β_1AR or β_2AR specific agonist (1 μM) for 15 min at 37 °C. As illustrated in Fig. 3B and C, phospho-PKA level observed in the presence of isoproterenol and formoterol (1 μM) alone or in combination with increasing concentrations of L-779976 remained relatively comparable to control without any significant changes. These results indicate that PKA phosphorylation and cAMP inhibition are receptor specific and independently regulated process.

3.5. SSTR2 activation inhibits PKCα and PKCβ expression in H9c2 cells

PKC isozymes exert hypertrophic stimuli in cardiac cells and increased expression of PKCα is associated with heart failure [33]. Furthermore, the inhibitors of PKC have been implicated to ameliorate hypertrophy [33]. To examine whether SSTR2 mediated inhibition of cAMP/PKA was also involved in the regulation of PKC, the expression levels of PKCα and β were determined in the presence of L-779976 in time and concentration dependent manner. H9c2 cells were treated with L-779976 (10 nM) for 0–30 min and with different concentrations (1–20 nM) for 15 min at 37 °C. As shown in Fig. 4A, the expression of PKCα and β was not changed in cells either treated with different concentrations or times with L-779976.

To assess the potential role L-779976 on isoproterenol and formoterol mediated changes in PKCα and β, H9c2 cells were treated with different concentrations of L-779976 (1–50 nM) with isoproterenol or formoterol (1 μM) for 15 min at 37 °C. As shown in Fig. 4B, PKCα expression was enhanced significantly in the presence of isoproterenol without any discernible changes in PKCβ expression. Increasing concentration of SSTR2 agonist L-779976 in combination with isoproterenol (1 μM) resulted in inhibition of PKCα in a concentration dependent manner when compared to control as well as isoproterenol treated cells. In contrast, PKCβ expression was comparable to control in cells treated with isoproterenol alone

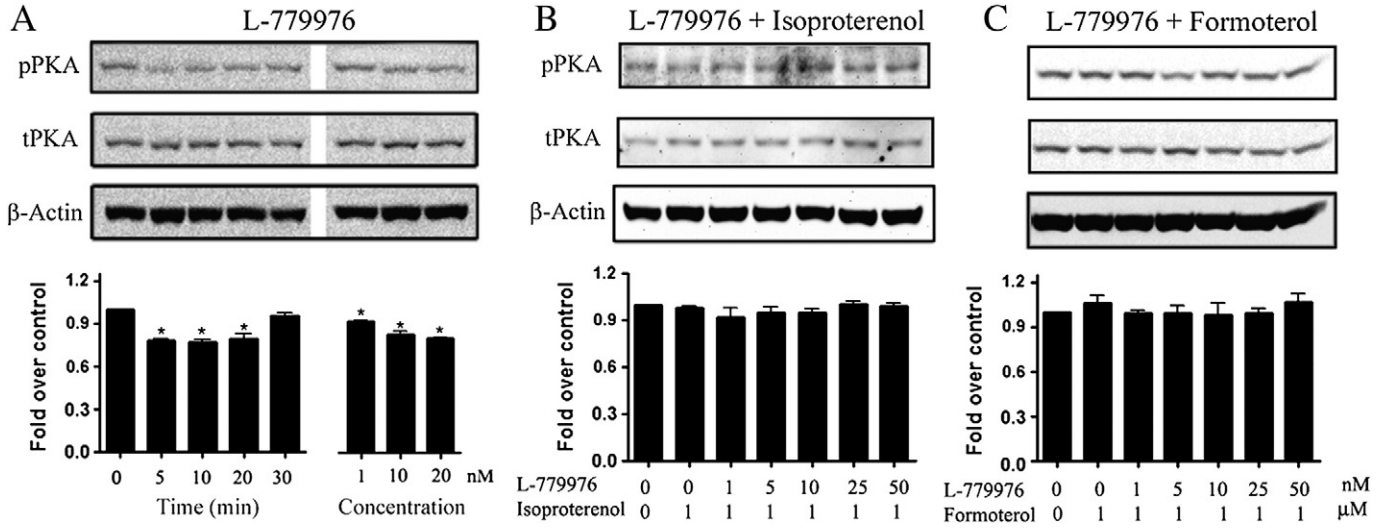

Fig. 3. Effect of SSTR2 agonist on isoproterenol and formoterol activated PKA. H9c2 cells were treated with SSTR2 agonist L-779976 (10 nM) for 0–30 min or with different concentrations (1–20 nM). Cells were also treated in combination with β-AR agonist (1 μM) in the presence of different concentrations of SSTR2 agonist (0–50 nM) as indicated for 15 min at 37 °C. (A) SSTR2 specific agonist inhibits PKA phosphorylation in time or concentration dependent manner. (B and C) The status of phospho-PKA was comparable to control in the presence of isoproterenol and formoterol alone or in combination with SSTR2 agonist. Data represents three independent experiments and analyzed by using ANOVA and post-hoc Dunnett's test to compare against basal (*, $p < 0.05$).

or in combination with SSTR2 selective agonist without any significant changes at different concentrations (Fig. 4B, bottom panel).

As shown in Fig. 4C, PKCα level was comparable to basal in cells treated with formoterol (1 μM) with or without L-779976. Conversely, L-779976 inhibited formoterol induced activation of PKCβ in a concentration dependent manner (Fig. 4C, bottom panel) significantly in comparison to control and formoterol treated cells. Interestingly, these observations uncovered distinct effects of β_1 and β_2AR activation on PKC isoforms. Most importantly, SSTR2 selective agonist inhibits changes in PKC isoforms induced in AR specific manner.

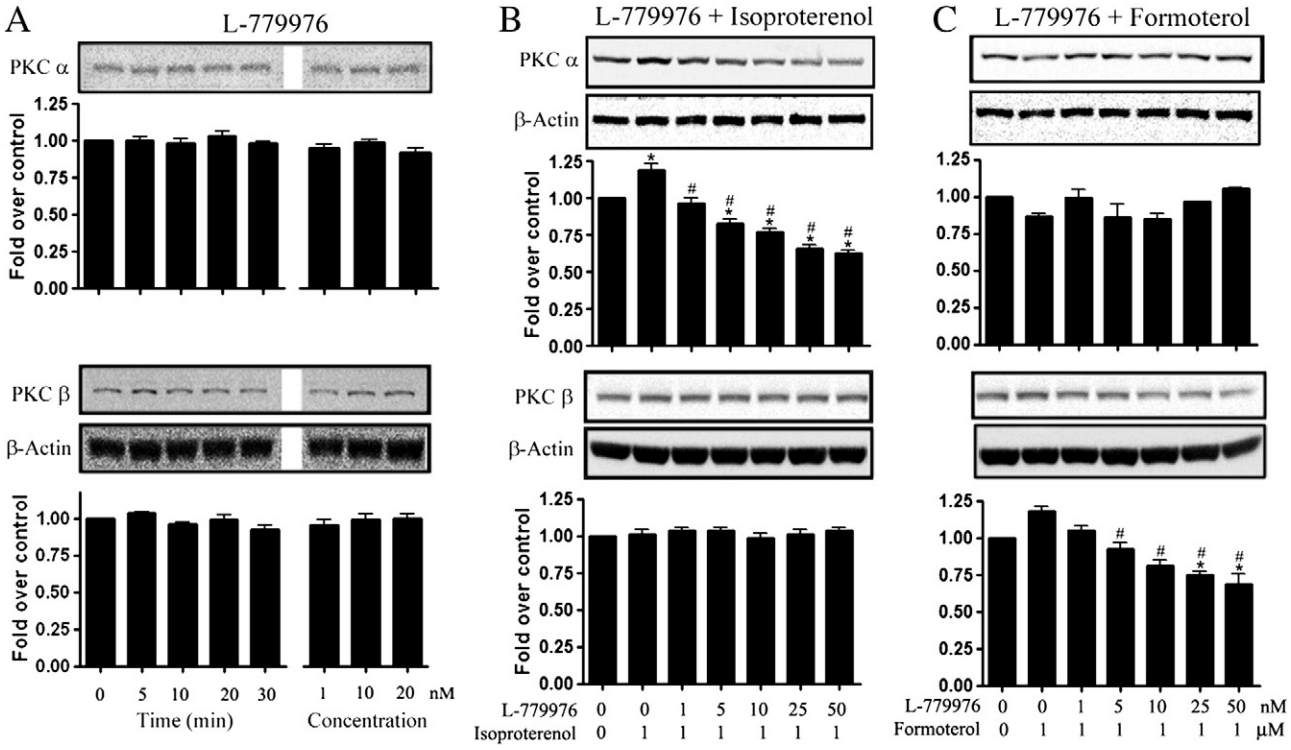

Fig. 4. Differential regulation of PKCα or β isoforms by β-ARs in H9c2 cells is inhibited by SSTR2 agonist. H9c2 cells were treated with L-779976, isoproterenol and formoterol as described in Fig. 3. Cells treated with different concentrations or times in the presence of SSTR2 agonist L-779976 displayed no significant effect on PKC (α or β) expression (A). In comparison to control, isoproterenol treatment enhanced the expression of PKCα (B upper panel) with no significant effect on PKCβ (B lower panel). Note the concentration dependent inhibition of isoproterenol induced PKCα expression in the presence of SSTR2 agonist significantly different from control and isoproterenol treatment. (C) In contrast, formoterol treatment enhanced PKCβ expression with no significant effect on PKCα. Note the significant inhibitory effect of SSTR2 in modulation of formoterol induced expression PKCβ (C, lower panel) without any effect on PKCα (C, upper panel). Data represents three independent experiments and data analysis was done by using ANOVA and post-hoc Bonferroni's test to compare against basal (*, $p < 0.05$) or β-AR agonist treatment alone (#, $p < 0.05$).

3.6. SSTR2 inhibits isoproterenol and formoterol enhanced calcineurin activity in H9C2 cells

Like cAMP/PKA/PKC, the activity of calcineurin in cardiac tissue and cells is also regulated by Ca^{2+}. To ascertain the role of SSTR2, the phosphatase activity of calcineurin and its expression was determined in H9c2 following treatment with SSTR2 and β-ARs specific agonists isoproterenol and formoterol. As shown in Fig. 5A, the phosphatase activity of calcineurin was inhibited by ~40% in the presence of SSTR2 specific agonist in comparison to control. Isoproterenol and formoterol enhanced calcineurin phosphatase activity by ~1.5 and ~1.25 folds respectively when compared to control. SSTR2 agonist in combination with isoproterenol and formoterol abrogated calcineurin phosphatase activity by ~50–60% which was comparable to L-779976 treatment alone whereas significantly different in comparison to control.

To further correlate the phosphatase activity, calcineurin expression was determined by Western blot analysis following treatments with receptor specific agonist in H9c2 cells. As illustrated in Fig. 5B, in comparison to control, calcineurin expression was inhibited upon treatment with L-779976. In contrast, calcineurin expression was enhanced significantly in cells treated with isoproterenol and formoterol alone. Consistent with the phosphatase activity, β-AR mediated increased expression level of calcineurin was significantly inhibited in the presence of L-779976 when used in combination with isoproterenol and formoterol. These results indicate that β-AR activated calcineurin expression and phosphatase activity are diminished upon activation of SSTR2 due to the expected inhibition of Ca^{2+} influx in H9c2 cells.

3.7. SSTR2 mediated NFAT phosphorylation and inhibition of its nuclear translocation

Calcineurin mediated NFAT dephosphorylation and its nuclear translocation have been linked in propagating hypertrophy. We sought to determine whether SSTR2 mediated inhibition of β-AR induced calcineurin expression and phosphatase activity also blocks NFAT dephosphorylation and its nuclear translocation. H9c2 cells were treated with SSTR2 specific agonist in time (0–30 min) and concentration (1, 10 and 20 nM) dependent manner at 37 °C and processed to determine the status of NFAT phosphorylation. As shown in Fig. 6A, cells exposed to L-779976 exhibited significant increase of NFAT phosphorylation in time and concentration dependent manner. As shown, the maximal activation of NFAT was observed at 5 and 10 min treatments with SSTR2 agonist (10 nM) or in the presence of 10 nM concentration of SSTR2 agonist for 15 min treatment.

To determine whether SSTR2 modulates β_1 and β_2AR induced NFAT phosphorylation, H9c2 cells were treated with different concentrations of L-779976 (1–50 nM) with isoproterenol or formoterol (1 μM). As shown, the NFAT phosphorylation was inhibited upon treatment with isoproterenol and formoterol alone (Fig. 6B and C). Furthermore, L-779976 ameliorates isoproterenol and formoterol induced inhibition of NFAT phosphorylation in concentration dependent manner and resulted in significant increase of phospho-NFAT levels when compared to control and cells treated with isoproterenol and formoterol alone (Fig. 6C). Interestingly, L-779976 mediated changes in the phospho-NFAT levels in the presence of formoterol were more pronounced in comparison to isoproterenol treatment.

3.8. SSTR2 agonist block NFAT nuclear translocation

To determine whether SSTR2 mediated activation of NFAT phosphorylation is also associated with the inhibition of NFAT nuclear translocation, H9c2 cells were processed for nuclear expression of NFAT. As shown in Fig. 6, control (D) and cells treated with L-779976 (E) displayed predominant cytoplasmic expression of NFAT. Conversely, cells treated with isoproterenol (F) and formoterol (G) resulted in enhanced nuclear localization of NFAT, which was significantly inhibited in the presence of SSTR2 agonist (H and I). Nuclear translocation of NFAT was relatively higher upon treatment with isoproterenol when

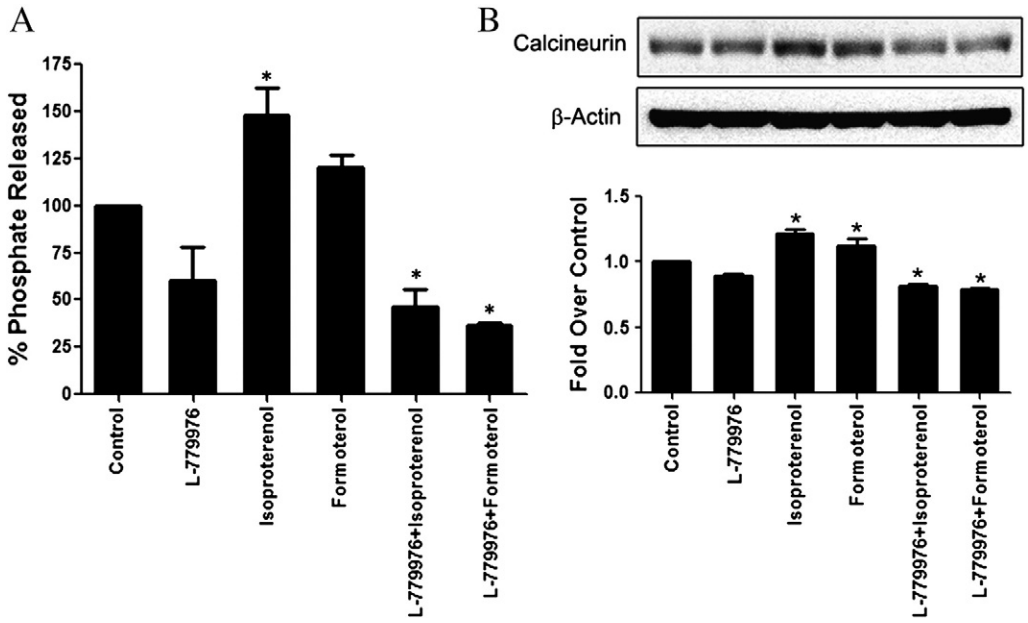

Fig. 5. SSTR2 negatively regulates calcineurin activity and expression in H9c2 cells. H9c2 cells were treated with SSTR2 agonist L-779976 (10 nM) for 30 min and cell lysate was processed for calcineurin phosphatase activity as described in the Materials and methods section. (A) Phosphatase activity was enhanced in the presence of isoproterenol or formoterol treatments whereas SSTR2 agonist exhibited inhibition of phosphatase activity. Note the significant effect of SSTR2 agonist on inhibition of calcineurin activity induced in the presence of isoproterenol or formoterol. (B) To determine the expression level of calcineurin, lysate prepared from control and treated cells was processed for Western blot analysis. Note the inhibition of β-AR mediated enhanced expression of calcineurin immunoreactivity upon SSTR2 agonist treatment alone or in combination with β-AR agonist. Data represents three independent experiments and data analysis was done by using ANOVA and post-hoc Dunnett's test to compare against basal (*, $p < 0.05$).

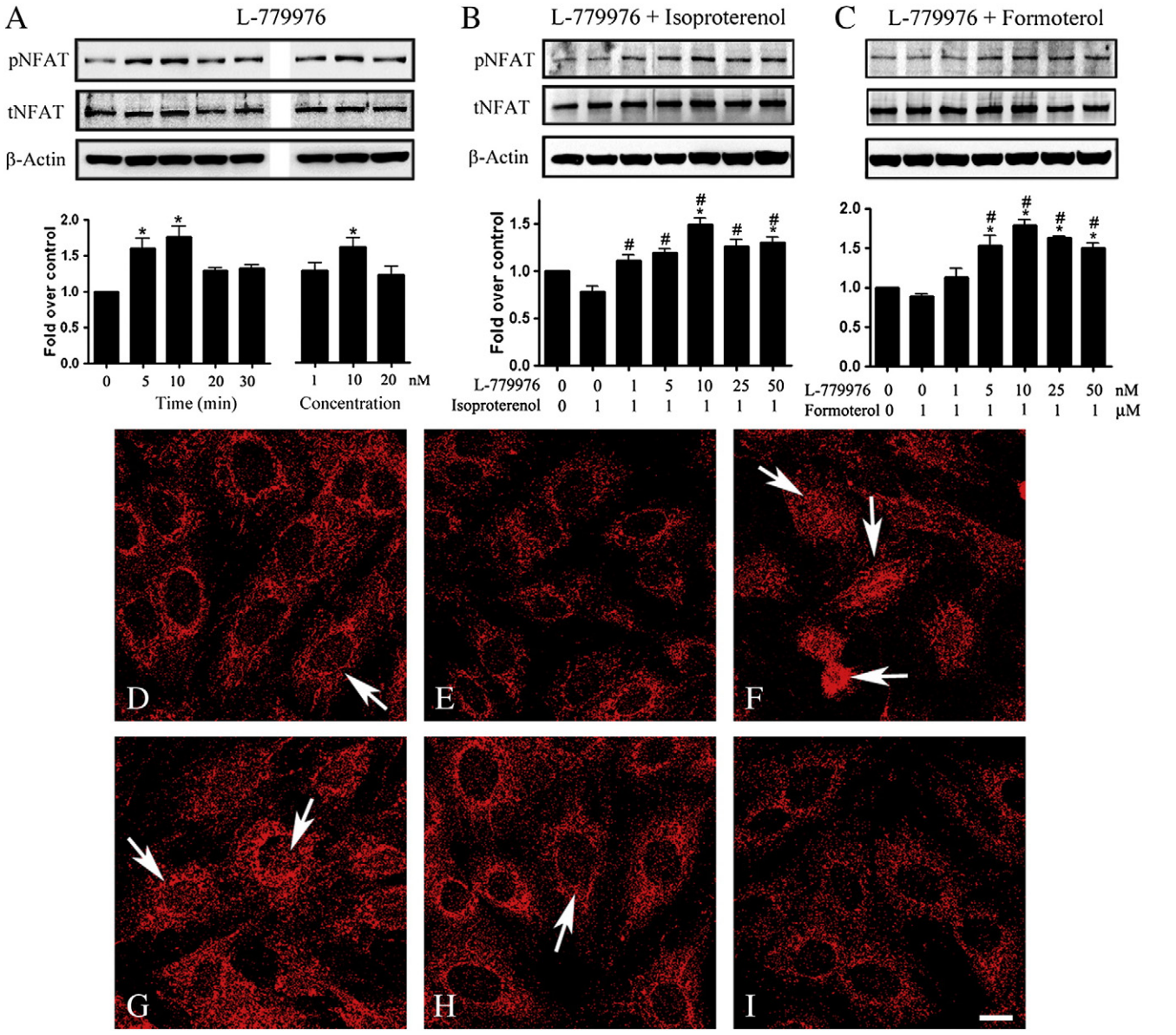

Fig. 6. β-ARs induced dephosphorylation and nuclear translocation of NFAT is blocked in the presence of SSTR2 agonist. H9c2 cells were treated with SSTR2 agonist (10 nM) for 0–30 min as well as with different concentrations (1–20 nM) for 15 min at 37 °C. SSTR2 agonist enhanced NFAT phosphorylation in time and concentration dependent manner with maximal effect at 10 min and 10 nM respectively (A). In comparison to control, isoproterenol or formoterol treatment alone elicits NFAT dephosphorylation (B and C). Increasing concentration of SSTR2 agonist L-779976 blocked isoproterenol or formoterol induced NFAT dephosphorylation and significantly enhanced phospho-NFAT levels (B and C) (*, $p < 0.05$ against basal and #, $p < 0.05$ against β-AR agonist treatment alone; n = 3). (D and E) To determine NFAT nuclear translocation, immunocytochemistry was performed in control and treated H9c2 cells. Control and cells treated with SSTR2 agonist displayed weak to negligible nuclear expression of NFAT. Note the increased nuclear expression of NFAT upon β_1AR or β_2AR activation (panels F and G). SSTR2 agonist in combination with isoproterenol (H) or formoterol (I) significantly blocked NFAT nuclear translocation. Arrows in representative panels indicate nuclear expression of NFAT. Scale bar = 10 μm.

compared to formoterol. These data suggest that SSTR2 inhibits NFAT dephosphorylation and its nuclear translocation and attest to the therapeutic implication of SSTR2 in abrogating NFAT induced cardiac hypertrophy.

3.9. SSTR2 activation inhibits isoproterenol induced cell size and protein content in H9c2 cells

In cardiac hypertrophy, increased cardiac pump function and decreased ventricular wall tension are associated with growth of individual myocytes. Furthermore, sustained and prolonged myocardial hypertrophy leads to heart failure. To ascertain the impact of changes in Ca^{2+} regulated signaling pathways, cell size and protein content were determined. H9c2 cells were treated with isoproterenol (5, 10 and 20 μM) alone or in combination with L-779976 (50 nM and 500 nM) for 48 h and processed for cell size analysis by employing two approaches i.e., morphological analysis and total protein estimation. As described in Fig. 7A, cells with or without SSTR2 specific agonist displayed comparable size without any noticeable changes at the different time points. In comparison, cells treated with isoproterenol displayed significant increase in the cell size over the increasing time period when compared to control or cells treated with SSTR2 agonist. Importantly, isoproterenol induced increase in cell size was abrogated in the presence of L-779976.

We also analyzed the concentration dependent effect of isoproterenol and SSTR2 agonist alone or in combination on total protein content per 10^6 cells using Bradford assay (Fig. 7B and C). Total protein content of H9c2 cells was significantly increased upon treatment with isoproterenol in concentration dependent manner whereas decreased in the presence of L-779976 (50 or 500 nM). Interestingly, isoproterenol (5,

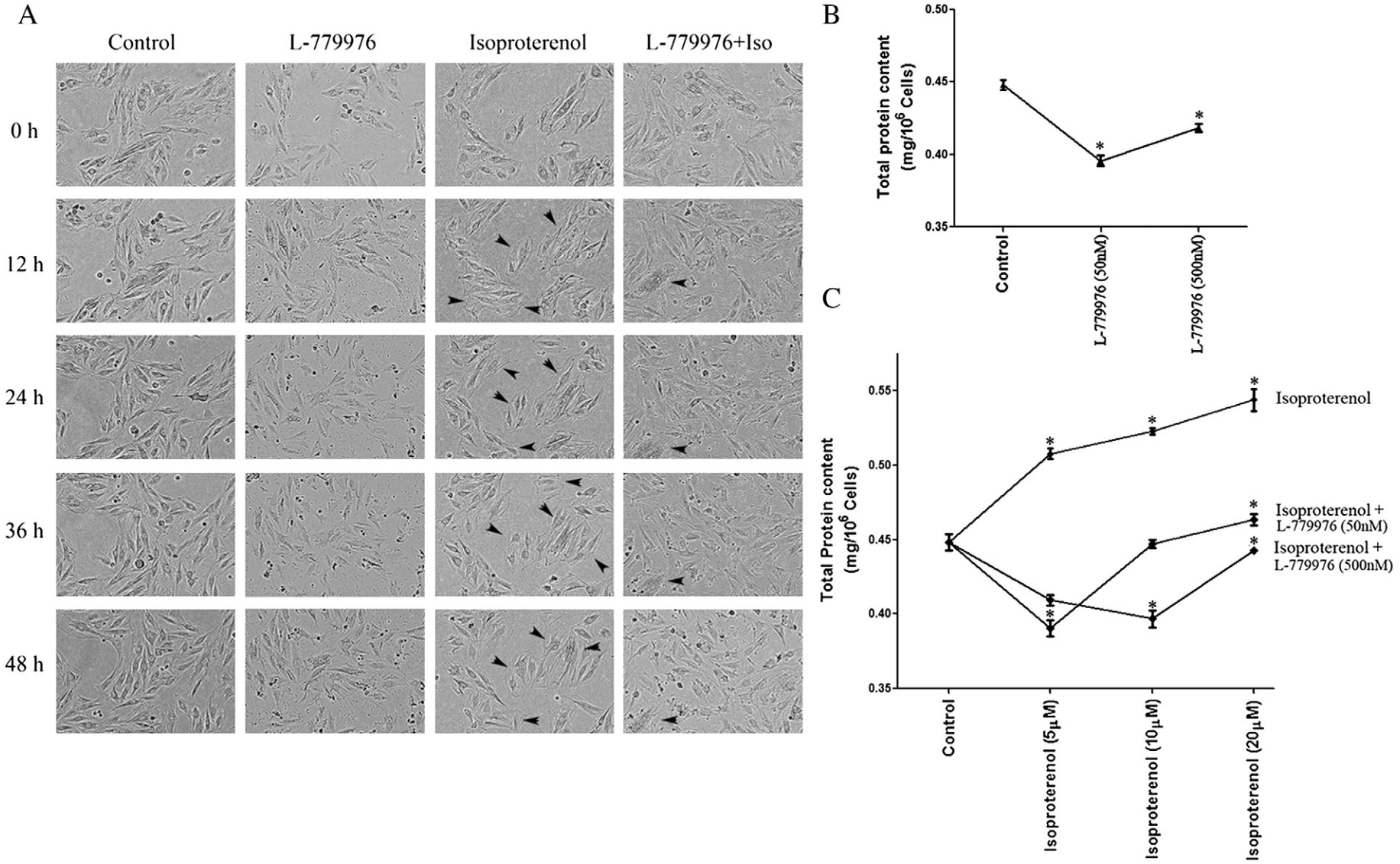

Fig. 7. Morphological changes in cell size upon receptor activation. (A) H9c2 cells were treated with isoproterenol (10 μM) in the absence or presence of L-779976 (500 nM) for 48 h. Cells were imaged by using the IncuCyte Zoom system every 2 h interval for 48 h using 10× objective. In comparison to control or L-779976, cells treated with isoproterenol displayed increased cell size (arrows). Isoproterenol mediated increase in cell size was blocked in the presence of SSTR2 selective agonist L-779976 and majority of cells displayed normal morphology. (B and C) Total protein content in H9c2 cells was determined by using Bradford assay following treatment with isoproterenol and SSTR2 specific agonist alone or in combination in the presence of 5% FBS. Decreased total protein content was observed upon treatment with SSTR2 specific agonist (50 or 500 nM) in comparison to control (B). Note that the presence of SSTR2 specific agonist (50 or 500 nM) significantly blunted the isoproterenol (5, 10, 20 μM) induced increase in the total protein content (C). Data are representative of three independent experiments (*, $p < 0.05$).

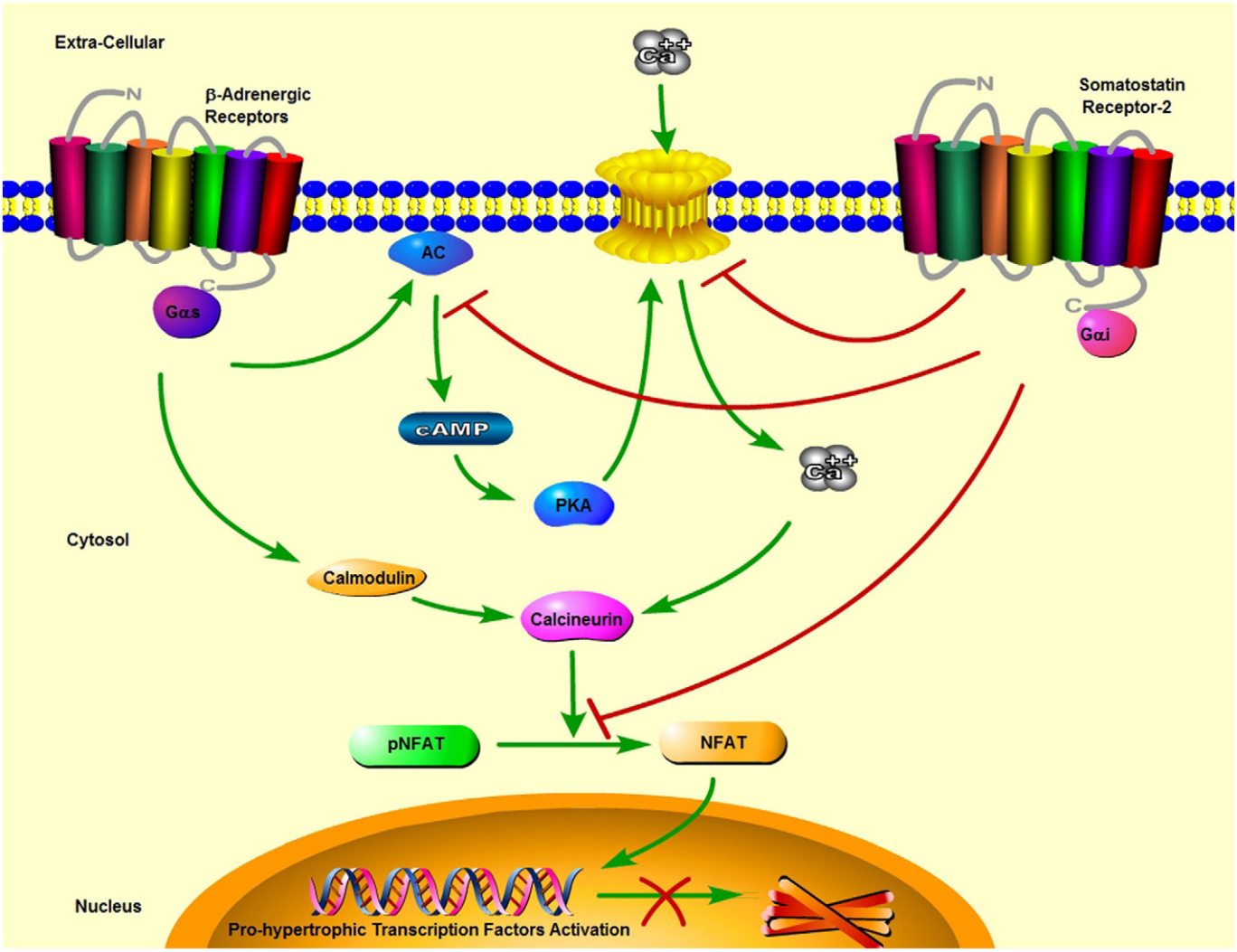

Fig. 8. Schematic representation of SSTR2 mediated regulation of β-ARs/Ca^{2+} dependent signaling pathways. SSTR2 activation inhibits β-AR mediated cAMP formation and Ca^{2+} influx in the cells and regulates calcineurin phosphatase activity. Inhibition of calcineurin phosphatase activity in turns maintains NFAT in phosphorylated state and abrogates its nuclear translocation. Overall, the regulation of these upstream checkpoints leads to the inhibition of pro-hypertrophic transcriptional factor activation in the nucleus. Figure was constructed by using the online pathway builder from Protein Lounge (http://www.proteinlounge.com).

10 and 20 μM) mediated increase in the total protein content was significantly blunted in the presence of SSTR2 specific agonist (50 or 500 nM) (B and C). These results suggest that SSTR2 mediated inhibition hypertrophy might be exploited as therapeutic intervention in cardiac complications or heart failure.

4. Discussion

Deregulation of Ca^{2+} associated interconnected complex signaling cascades such as cAMP/PKA/PKC and nuclear transcription factors like cAMP response element binding protein (CREB) and NFAT plays a critical role in cardiac hypertrophy and heart failure [34–37]. Most importantly, activation of cAMP/PKA in the presence of β-ARs supports the receptor specific role in myocyte contractility. Furthermore, inhibition of calcineurin and NFAT dephosphorylation with consequent blockade of NFAT nuclear translocation serves as critical step in preventing cardiac complications. We recently described that SST modulates β_1AR mediated key signaling pathways associated with cardiac hypertrophy in H9c2 cells [19]. However, SSTR subtype that might regulate such crucial role of SST in cardiac cells is not well understood. In the present study, we delineate the role of SSTR2 in modulation of β-AR mediated signaling pathways which are associated with increased Ca^{2+} and cardiac hypertrophy (Fig. 8). Our results provide direct evidence that SSTR2 negatively regulates β-AR induced signaling. To our knowledge this is the first comprehensive description delineating the role of SSTR2 in the regulation of Ca^{2+} associated intracellular signaling cascades which play detrimental roles in cardiac complications.

Previous studies have also demonstrated that SST and its analogs Octreotide and Lanreotide impart cardio-protective effect in the left ventricular hypertrophy (LVH) [23,25,38,39]. The specific SSTR subtype associated with cardiac protection is not well understood however, Octreotide exhibits high affinity binding to SSTR2 whereas, SST and its analog mediated regulation of Ca^{2+} are specifically arbitrated by SSTR2 [28]. These observations support that SSTR2 is the prominent receptor subtype of the family which might play beneficial role in cardiac complications. We here demonstrate that SSTR2 colocalized with β_1AR and β_2AR in H9c2 cells and is expressed in β_1AR and β_2AR immunoprecipitates. The weak expression of SSTR2 in β_1AR immunoprecipitate upon isoproterenol treatment might indicate either preferential homodimerization of β_1AR. Furthermore, β_1AR heterodimerization with β_2AR may trigger receptor internalization.

SSTRs are involved in the regulation of Ca^{2+} inward currents via cAMP dependent and independent pathways and act directly on high-voltage-dependent Ca^{2+} channels via $G\alpha_0$ protein [15,40–43]. Previous studies have shown increased calcium influx in H9c2 cells in response to β-AR agonists [44]. We found that SSTR2 agonist negatively regulates

forskolin induced formation of cAMP in response to β-ARs. Moreover, SSTR2 mediated inhibition of cAMP was enhanced in the presence of β-AR antagonist. In contrast, SSTR2 agonist alone or in combination with β-AR selective agonists maintained PKA phosphorylation comparable to control in time and concentration dependent manner. We have previously demonstrated that in heterologous system (β_1AR/SSTR5 cotransfected HEK-293 cells), SSTR5 specific agonist treatment significantly enhanced PKA phosphorylation [30]. These results uncover distinct G-proteins involved in heterologous system when compared to the cells expressing these receptors endogenously. Whether or not SSTR2 functions in a similar manner like SSTR5 with β-ARs in HEK-293 is not known and needs to be determined. Our results indicate that the regulation of cAMP and PKA phosphorylation are two independent processes. If FSK imparts such distinct effects on cAMP regulation and downstream signaling pathways including PKA phosphorylation warrants further studies.

Previous studies have shown that certain markers of hypertrophy like mRNA level of atrial natriuretic peptide (ANP), increased protein synthesis, protein to deoxyribonucleic acid (DNA) ratio, and cell size were induced upon PKCα activation [45,46]. Also, over-expression of PKCα results in cardiac dysfunctions [47]. Like PKCα, another isoform namely PKCβ is also critical in promoting hypertrophic growth in cardiomyocytes [48,49]. In hypertension-induced heart failure rats, increased PKCβ levels were observed whereas selective inhibition of PKCβ improved cardiac function [48,49]. Angiotensin-receptor antagonist decreases PKCβ activation and results in improved cardiac hypertrophy [33,49]. Moreover, intracellular Ca^{2+} and lipid mediated signaling activate PKC and consequently impaired cardiac function [50]. In agreement with these observations, results presented here describe attenuation of β-AR mediated increased PKCα and β expression in the presence of SSTR2 agonist in concentration dependent manner. Most importantly, SSTR2 specific agonist does not exert any significant effect on PKCα and β expression when used alone. These results strongly emphasize the role of receptor heterodimerization in H9c2 cells and attest to the distinct role of receptor complex in comparison to native receptors. Furthermore, our results also uncovered selectivity and specificity of SSTR2 in the regulation of β-AR mediated expression of PKC isoforms and its inhibition in either condition. Whether direct interactions between PKC isoforms exert any plausible role on receptor mediated signaling pathways is not well understood. Taken in consideration, SSTR2 mediated inhibition of PKCα and β expression attests its beneficial role in cardiac complication and might serve as a potential therapeutic target.

SSTR2 mediated inhibition of cAMP/PKA and PKCα/β leads us to speculate that Ca^{2+} dependent calcineurin activity, NFAT dephosphorylation and its nuclear translocation are interdependent events regulated by myriad of factors. In cardiac tissues, increased Ca^{2+} directly or indirectly via activation of calcineurin plays an essential role in hypertrophy and targeting this pathway has emerged as a possible therapeutic approach [51]. To further support these observations, we here describe that SSTR2 regulates calcineurin expression and its activation (phosphate release) in H9c2 cells alone or in the presence of isoproterenol. The inhibition of PKC, p38, and Gi resulted in decreased basal NFAT transcription [52] whereas ERK1/2 has been shown to activate NFAT in T-lymphocytes and in COS cells [53]. Consistent with these observations, we observed enhanced phospho-NFAT levels upon SSTR2 agonist treatment in combination with either isoproterenol or formoterol and provided biochemical and morphological evidences that SSTR2 plays a critical role in blocking NFAT dephosphorylation. Nuclear translocation of NFAT by isoproterenol and formoterol was also significantly abolished upon activation of SSTR2.

Cardiac hypertrophy involves remodeling of myocytes at both molecular and cellular levels [54]. Specifically in pathological cardiac hypertrophy, these changes result in lengthening of myocytes and dilation along with impaired cardiac functions [54]. Left ventricular remodeling is dependent upon several factors including severity of the pathological condition in patients and also to the extent of isoproterenol treatment in the experimental animals [55]. Here, we demonstrate that SSTR2 agonist treatment in H9c2 cells resulted in significant blockade of isoproterenol mediated cell size and protein content. Although further studies are warranted, however the potential role of SSTR2 as a negative regulator of Ca^{2+} mediated signaling and hypertrophy cannot be underestimated. Since, SSTR subtypes function in concert as heterodimers, the role of other SSTR subtypes alone or in combination with SSTR2 cannot be avoided from discussion and further studies are in progress in this direction. Taken in consideration, targeting multiple steps in the regulation of Ca^{2+} associated signaling regardless of molecular mechanism involved, our results implicate SSTR2 as a pharmacological intervention in cardiac complications or heart failure. We postulate that activating SSTR2 with concomitant blockade of β_1AR in the presence of specific antagonist might serve as a potential therapeutic approach in the regulation of pathological hypertrophy.

Acknowledgements

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